Gene Expression Profiling Soybean Stem Tissue Early Response to *Sclerotinia sclerotiorum* and In Silico Mapping in Relation to Resistance Markers

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Abstract

White mold, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, can be a serious disease of crops grown under cool, moist environments. In many plants, such as soybean [*Glycine max* (L.) Merr.], complete genetic resistance does not exist. To identify possible genes involved in defense against this pathogen, and to determine possible physiological changes that occur during infection, a microarray screen was conducted using stem tissue to evaluate changes in gene expression between partially resistant and susceptible soybean genotypes at 8 and 14 hours post inoculation. RNA from 15 day-old inoculated plants was labeled and hybridized to soybean cDNA microarrays. ANOVA identified 1270 significant genes from the comparison between time points and 105 genes from the comparison between genotypes. Selected genes were classified into functional categories. The analyses identified changes in cell-wall composition and signaling pathways, as well as suggesting a role for anthocyanin and anthocyanidin synthesis in the defense against *S. sclerotiorum*. In-silico mapping of both the differentially expressed transcripts and of public markers associated with partial resistance to white mold, provided evidence of several differentially expressed genes being closely positioned to white mold resistance markers, with the two most promising genes encoding a PR-5 and anthocyanidin synthase.

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THE FUNGAL PATHOGEN *Sclerotinia sclerotiorum* (Lib.) de Bary is an important pathogen that infects a wide variety of vegetables, ornamentals, and field crops causing a disease known as either white mold or Sclerotinia stem rot. Plants susceptible to this pathogen encompass 75 families, 278 genera, and 408 species (Boland and Hall, 1994). One of the main pathogenic factors of *S. sclerotiorum* is oxalic acid (OA) (Godoy et al., 1990). Recently, Kim et al. (2008) provided evidence to the hypothesis that OA induces cell death to help the pathogen on initial infection of the tissue. Additional mechanisms of action for the secreted OA have been proposed, and other pathogen-secreted factors such as cell-wall degrading enzymes and polygalacturnases have also been implicated as pathogenicity factors (Cessna et al., 2000; Favaron et al., 2004;

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; CCR, cynnamoyl CoA reductase; CT, cycle threshold; Cy3, cyanine 3 fluorescent dye; Cy5, cyanine 5 fluorescent dye; ERF1, ethylene responsive factor1; EST, expressed sequence tag; ET, ethylene; FDR, false discovery rate; glowess, global lowess; hpi, hours post inoculation; IFRs, isoflavone reductase homologs; JA, jasmonic acid; LDOX, leucoanthocyanidin dioxygenase; lowess, locally weighted linear regression; OA, oxalic acid; PR, pathogenesis-related; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; QTL, quantitative trait loci; rlowess, Regional lowess; ROS, reactive oxygen species; R, partially resistant soybean PI 194639; S, susceptible soybean 'Williams 82'; Satt, satellite repeat sequence; SSR, simple sequence repeat.

Guimaraes and Stotz, 2004; Marciano et al., 1983; Riou et al., 1991; Sperry and Tyree, 1988).

In soybean [*Glycine max* (L.) Merr.], Sclerotinia stem rot is very difficult to control. Resistance to this pathogen is quantitative and no fully resistant soybean genotype has been found to date. Agronomic practices like rotation and tillage (Mueller et al., 2002a), fungicides (Mueller et al., 2002b), and the use of partial resistance (Hoffman et al., 2002) have all been evaluated, but none have been completely effective. A number of studies have tried to define the genetic basis of soybean resistance to *S. sclerotiorum* (Arahana et al., 2001; Kim et al., 1999; Kim and Diers, 2000; Vuong and Hartman, 2003). One of these studies used the partially resistant PI 194639 (Vuong and Hartman, 2003). A later study on this genotype used a multivariate model to conclude that all quantitative trait loci (QTL) in PI 194639 that were deemed significant explained 27% of the observed phenotypic variation measured (Vuong et al., 2008). Recently, lignin content of soybean has been proposed to have an inverse correlation with resistance as partially resistant soybean accessions were found to have lower lignin contents (Peltier et al., 2009).

Identification of genes that are differentially expressed during soybean defense against *S. sclerotiorum* will contribute to the understanding of the physiology and molecular basis of defense. The information from soybean response to *S. sclerotiorum* may lead to strategic engineering of effective resistance, such as the development of the *S. sclerotiorum* resistant soybean transgenic line carrying an oxalate oxidase gene from wheat (Cober et al., 2003). The molecular information may also provide candidate genes for QTL mapping and marker-assisted breeding. The introduction of microarray technology in recent years has provided a tool to screen for differential regulation of thousands of genes simultaneously in a single experiment.

Three independent studies have described the *S. sclerotiorum–Brassica napus* pathosystem at the genomic level utilizing *Arabidopsis thaliana* microarray platforms. Liu et al. (2005) screened the response of leaf tissue from a partially resistant *B. napus* cultivar Ning RS-1 vs. the susceptible breeding line H5200 utilizing cDNA microarrays consisting of 9216 expressed sequence tags (ESTs). A later study compared leaf tissue of inoculated vs. noninoculated susceptible *B. napus* var. Wager with an oligonuclotide microarray platform consisting of 26,000 *A. thaliana* genes (Yang et al., 2007). Partially resistant *B. napus* line RV289 vs. the highly susceptible cultivar Stellar were compared with the same 26,000 *A. thaliana* oligonucleotide platform (Zhao et al., 2007).

Soybean cDNA microarrays (Vodkin et al., 2004; Vodkin et al., 2007) and Affymetrix gene expression chips have been used to study soybean response to microbes. Several soybean-pathogen systems were studied using one of these platforms, including *Phytophthora sojae* (Moy et al., 2004), *Pseudomonas syringae* (Zou et al., 2005), *Phakopsora pachyrhizi* (Panthee et al., 2009; van de Mortel et al., 2007), *Bradyrhizobium japonicum* (Brechenmacher et al., 2008), and soybean mosaic virus (Bilgin et al., 2008).

The main objectives of this study were to investigate the molecular mechanisms by which soybean responds to *S. sclerotiorum* and to try to identify the physiological basis of partial resistance. These two objectives were addressed by performing a microarray study comparing gene expression at two time points during the period of early infection: 8 and 14 hours post inoculation (hpi); comparing the partially resistant (R) soybean PI 194639 with the susceptible (S) genotype 'Williams 82' and mapping to the soybean genome to identify differential cDNA in close proximity to known soybean resistance markers for *S. sclerotiorum*.

MATERIALS AND METHODS

Plant Growth, Inoculation, and Sampling

Seeds of R PI 194639 and S Williams 82 soybean were planted in SunshineMix (SunGro, Vancouver, BC) LC1 soil medium in 3.5 inch square pots. The plants were grown in a growth chamber at 23°C with a photoperiod of 12/12 (day/night) and a light intensity of ~180 µmol photons m^{-2} s^{-1} for a period of 15 days until the unifoliates were fully expanded and the first trifoliates were unfolded. Fungal cultures from *S. sclerotiorum* isolate 105HT were started 24 hours in advance by sub-culturing actively growing edges of fungal colonies from stock cultures onto potato dextrose agar. Just before inoculation, 5 mm diameter plugs of agar with growing mycelium were cut from the edges of colonies using a cork borer.

Twenty-four hours before inoculation (14th day post planting), the photoperiod was changed to 16/8 (day/night) to extend the available light hours for inoculation and sampling. Prior to the second hour of daylight, stems of all plants were inoculated with the prepared agar plugs as previously described (Vuong et al., 2004) with minor modifications. Plants were cut horizontally at the stem with a clean straight-edge razor under the node of the first trifoliate. Agar plugs were placed on the fresh wound with the mycelial side touching the cut stem. Two humidifiers (TRION 500; Sanford, NJ) were turned on immediately after inoculations and the infected plants were left under near 100% humidity until the last sampling time. After inoculation and during the first three hours, the chambers were checked every 30 minutes to ensure no agar plugs had slipped off. If so, new agar plugs were replaced. Approximately 10% of the plugs needed replacement, and no plugs needed to be replaced after the first hour. Samples were taken at 8 and 14 hpi by cutting the top 2.5 cm of stem. Thirteen 2.5 cm stem sections were randomly collected and pooled per genotype per time. Control samples (noninoculated, freshly-cut stems from 15 day-old seedlings) were also collected. Additional intact plants from both susceptible and resistant genotypes were left in the growth chambers for five days to confirm the expected phenotypic responses.

All inoculation and sampling procedures were performed inside the growth chamber to minimize disturbance to the plants which could result in the expression of genes not related with the inoculation. After cutting, the samples were quickly placed into Kapack pouches (Kapak, LLC., Minneapolis, MN) and frozen in liquid nitrogen within 30 seconds of cutting. Samples were transported under liquid nitrogen to the laboratory and stored at 80°C. The experiment was repeated three times, each at a different date with new inoculum, to obtain three independent biological replications.

RNA Isolation, Dye Incorporation, and Microarray Hybridization

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) following manufacturer's protocol coupled with Phase Lock Gel (Brinkmann Instruments, Inc., Westbury, NY) and further purified through Qiagen RNeasy columns (Qiagen, Valencia, CA). RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA quality was determined by combination of spectrophotometry and gel electrophoresis with a BioAnalyzer 2100 (Agilent Technologies, Palo Alto, CA). RNA labeling and microarray procedures closely followed that published by Zou et al. (2005). High quality RNA was reverse transcribed into cDNA with SuperScript III Reverse Transcriptase enzyme (Invitrogen, Carlsbad, CA) in the presence of aminoallyl-dUTP followed by hydrolysis of the RNA with strong base and removal of the unincorporated dNTPs and other salts and contaminants with a Qiaquick PCR purification kit (Qiagen) with TRIS-free solutions. Samples were coupled to either Cyanine 3 (Cy3) or Cyanine 5 (Cy5) fluorescent dyes (Perkin-Elmer, Foster City, CA), purified, mixed according to the loop design for each of the experiments (Fig. 1), suspended in hybridization buffer, and applied onto microarray slide libraries 18kA and 18kB (Vodkin et al., 2004; Vodkin et al., 2007). The slides were incubated within a sealed hybridization chamber submerged in a water bath in the dark at 42°C for a period of 48 hours. After incubation, a series of washes were performed on the slides to remove non-binding probe (Zou et al., 2005). Each biological replication was hybridized separately following the same procedure. A total of 36 slides were hybridized: 24 slides corresponding to the loop design in Fig. 1A (four of the 18kA library and four of the 18kB library, with three replications), and 12 slides for control samples (Fig. 1B) (two genotypes, two slide libraries, with three replications).

Image Acquisition and Processing

After washings, the slides were dried by centrifugation and scanned in a laser scanner (Scan ArrayExpress, Perkin-Elmer, Foster City, CA) as previously described (Zou et al., 2005). Images were obtained for each of the two laser channels (Cy3 and Cy5) for each slide. Spot intensities were quantified using GenePix Pro v. 4.1. (Axon, Milpitis, CA). Bad spots were flagged to exclude them

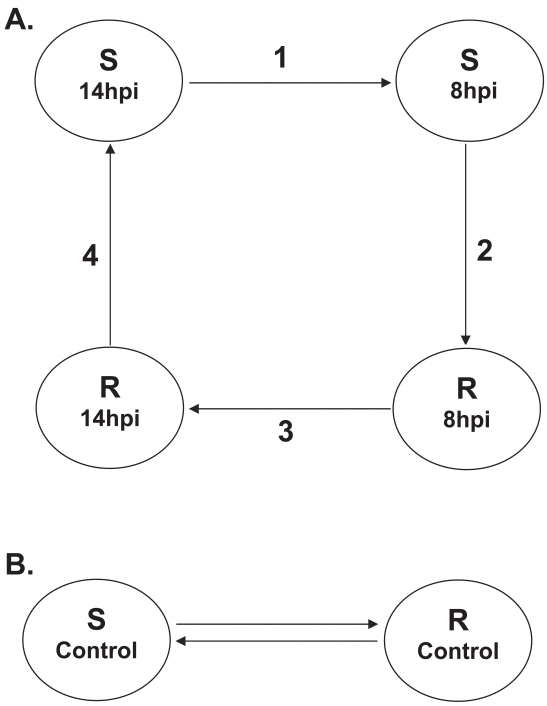

Figure 1. Experimental design. Panel A. Hybridizations of slides followed a "loop design". Panel B. Control noninoculated samples were directly compared including a dye swap. In each diagram, the balls correspond to samples and each arrow corresponds to one slide. Samples in the arrowheads were labeled with Cy5 and samples in arrow tails were labeled with Cy3.

from the final computations. A file (.gpr) was obtained for each of the slides containing all the data and scanned image statistics for each spot. These tables were parsed into one table containing only the median of the individual pixel-intensities per spot without background correction for each channel (Cy3 and Cy5) of each slide using an in-house PERL script. At the same time all flagged spots were replaced by the median of the negative control gene (X13988, human myosin) to avoid the outliers that might affect subsequent analyses such as normalization.

Data Normalization and Analysis of Variance

Normalization

Data analysis was performed closely following the method previously described in Brechenmacher et al. (2008). Data were normalized using the R-MAANOVA package (Wu et al., 1995; Wu et al., 2003). Two normalization methods were tested, both based on the locally weighted linear regression (lowess) approach (Cleveland, 1979). The first was glowess (global lowess) that smoothes the scatter plot (R/G) vs. (R*G) for the entire array. Secondly, a rlowess (regional lowess) was used which smoothes the scatter plot of (R/G) vs. intensity R*G and grid location, thus providing correction for systematic spatial variation within the array that can be caused by many factors such as differences in the printing pens, hybridization conditions, uneven slide coating, nonhomogenous hybridization solution, etc. The data obtained with the rlowess method provided a better smoothing of the data compared to glowess

as determined by visually comparing the RI plots ($\log_2$ R/G vs. $\log_2$ R*G) after normalization for each array, and therefore was used for the final analyses. After normalization, the weak spots (those with median intensity lower than the average of the negative control X13899) were changed to the median of the negative control spots in efforts to negate the effect that their high variability might have on the analysis. The normalized fluorescence data was then used for analysis of variance followed by paired *t*-tests to find the statistical significance in induction or repression of the transcripts.

ANOVA

SAS software (v9.1, SAS Institute, Inc., Cary, NC) was used to give format to the data set and to run the ANOVA on a per gene basis. Analysis of variance was run on the data using the model:

$$Y_{ijk} = \mu + A_i + G_j + T_k + (GT)_{jk} + \varepsilon_{ijk}$$

Where: Y_{ijk} is the median of the signal intensity for individual spot and channel; μ is the average $\log_2$ fluorescent signal intensity; A_i is the random effect of the array; G_j is the effect of the genotype; T_k is the effect of sampling time; $(GT)_{jk}$ is the interaction between genotype and time effects and ε_{ijk} represents the residual error. $(GT)_{jk}$ was found to be non-significant with an overall *p*-value equal to 0.05 and was disregarded from the model.

The Dye effect and interactions between Array and Genotype (AG), Array and Time (AT) and the three way interaction between Array, Genotype, and Time (AGT) were not accounted for in the model as they are not of interest and would utilize degrees of freedom that could be used to estimate the error variance. Additionally, the Dye effect showed no significant difference in a preliminary analysis on the data and only few genes showed weak significant difference in Dye effect in previous experiments in the lab (data not shown). Presumably, normalization is enough to correct for the dye effects, making them not significant.

LSMEANS were computed and a *t*-test was run on all data to measure the significance of the difference for each gene in all possible pair-wise treatment comparisons. The estimate was later used to compute back the $\log_2$ intensity ratios and prepare a table of fold changes of all comparisons together with their corrected and raw *p*-values.

Two tables were prepared for further analysis: a table containing all genes with FDR corrected *p*-values <0.05 for the *t*-test comparing both varieties in either or both time points referred as the "between varieties" table, and a second table containing all genes with FDR corrected *p*-values <0.05 for the *t*-test comparing both time points in either or both varieties.

Identification of Constitutively Differentially Expressed Transcripts between Genotypes Using Control Samples

Control samples (0 hpi) were obtained following the same procedure as described above. Each of the PI 194639 samples were hybridized with a corresponding Williams 82 sample using dye swap (changing the assigned Cy dye) for a total of six slides per library.

The data was normalized as described above and a statistical analysis of differentially expressed genes was performed using SAS with the following model:

$$Y_{ij} = \mu + A_i + G_j + \varepsilon_{ij}$$

Where: Y_{ij} is the median of the signal intensity for individual spot and channel; μ is the average $\log_2$ fluorescent signal intensity; A_i is the random effect of the array, G_j is the effect of the genotype and ε_{ij} is the residual error.

Functional Category Classification

Genes found to be statistically significantly different in abundance were classified into functional categories according to their sequence homology to genes in public databases. The NCBI NR protein database was used in conjunction with TIGR TC annotations. Homologies with e-values $>10^{-7}$ or <13% identity were classified as "no hits". Genes that had considerably different annotations in both databases were classified as "miscellaneous-multiple annotations", and genes with annotation differences from the sequences obtained from the 5′ end compared to 3′ end were classified as "miscellaneous-possible chimeric" if the homologies were very strong for both 5′ and 3′ end sequences. Classification of transcripts was done from a plant-microbe interaction point of view. The criteria used for classification can be found in Table 1.

Quantitative Real Time RT-PCR

QRT-PCR was performed on selected genes to assess the quality of the microarray data. A group of cDNAs present on the microarray slides was selected for this analysis. The genes were initially selected from the two lists of differentially expressed genes generated from the statistical analysis: the "between genotypes" table and the "between time points" table with FDR *p*-value <0.05. The lists were narrowed down by selecting genes with fold change >2 up or down ($\log_2$ intensity ratio >1.00 or <–1.00) in either or both time points for the "between genotypes" table and selecting genes with fold change >5 up and down for the "between time points" table. A list of 83 genes was obtained, the list was organized into functional categories and genes were picked randomly from each of the categories.

QRT-PCR was performed using the remaining total RNA from one replication used in the microarray study, plus two new additional biological replications and three biological replicates of control samples (0 hpi). The RNA was purified with RNeasy Mini Kit (Qiagen, Valencia, CA) as described above with an additional step

Table 1. Summary of criteria used for gene functional classification.

Category	Subcategories	Examples
Cell wall	– Cellullose: synthesis/degradation – Hemicellullose: synthesis/degradation – Pectins: synthesis/degradation – Callose synthesis/degradation – Structural proteins – Other misc. enzymes	Cyclin and cyclin-like, cell division control proteins, beta-glucanase, xylan 1,4-beta-xylosidase, cellulose synthase, PMEs PGAs, PRP, HRGPs, UDP-sugar synthesis.
Cytoskeleton	– Actin – Kinesin – Microtubule-related	
Defense	– Apoptosis – PR proteins – R proteins	Apoptosis regulators, apoptosis inhibitors, beta-1,3-glucanase, chitinase, thaumatin, defensin, SNAKIN, Pto interacting, disease resistante with LRR domains.
DNA/RNA	– DNA processing – RNA processing – Gene regulation – Nuclear organization	Helix-loop-helix domains, most types of zinc fingers, histones, polymerases, helicases, others transcription factors, components of splicesome, capping enzymes and poly (A) polymerase.
Energy	– ATP – Electron transfer – Photosynthesis	ATPases Ferredoxins photosystems subunits, RUBISCO subunits, DAG protein, cytocromes
Lipids	– Fatty Acid biosynthesis, degradation, utilization – Eicosanoids and isoprenoids biosynthesis, utilization and degradation	HMG-CoA synthase, acetylCoA-Carboxylase
Membrane	– Integral to membrane (structural) – Transport – Vesicle mediated transport	Aquaporins, ABC transporters, ion channels, sugar transporters nitrate transporters, Chlathrin. TIM and TOM.
Miscellaneous	– Do not fit into any of the mentioned categories – Several possible functions. – Chimeric – Different annotations across databases	Cytochrome P450, Ankyrin, Anexin
No hits	No significant homology with any record on the databases. (E-value greater than 10^{-7} or percentage of similarity less than 13%)	
Oxidation	– Catalses – GST – Oxidases – Oxidoreductases – Peroxidases – SODC	
Primary metabolism	– Amino Acids metabolism – Carbohydrates metabolism – Nucleosides and nucleotides metabolism – Vitamins / Cofactors metabolism	
Protein	– Degradation and Inhibition of Degradation – Folding (include all chaperones and cyclophylins) – Localization – Modification (Includ all additions of small and large groups: methylation, acetylation, phosphorylation, glycosylation, lipidation, prenylation).Except ubiquitination which is classified as a degradation process. – Synthesis	Proteasome subunits, ubiquitin, metalloproteases, serine proteases, ubiquitin ligase, protease inhibitors, chaperones, heat sock proteins, ribosome subunits.
Secondary metabolism	– Alkaloid biosynthesis – General Phenylpropanoid pathway – Flavonoid biosynthesis – Lignin biosynthesis – Isoprenoid pathway (mevalonate pathway derived and non-mevalonate pathway) including: Terpenes, sesquiterpenes, diterpenes, polyprenols. Excludes the ones that can be classified as hormones and primary metabolites.	CHS/CHR, IFS, IFR, F3H, FLS, DFR, HID, COMT, 4CL CCoAOMT, F5H, CAD/SAD. HMG-CoA, mevalonate kinase, Farnesyl PP synthase, Geranylgeranyl PP synthase
Senescence	Known to be directly related with senescence	SRG1

Table 1. Continued.

Category	Subcategories	Examples
Signalling	– Calcium Signalling – G proteins – Hormones. (ABA, auxins, ethylene, citokinin, giberelic acid; involved in the synthesis of the molecules or in their respective signalling events) – Inositol (involved in the signalling pathway or only in the biosynthesis of inositol are included) – Kinases that are not specific or are not known to any of the signalling pathways mentioned. – Phosphatases that are not specific or are not known to any of the signalling pathways mentioned. – Receptors /sensors	EF hands domains, calmodulin, monomeric G proteins, heterotrimeric G proteins, ERF, myo-inositol-phosphate synthase, Ser/Thr kinase, MAP kinases
Stress	Any protein known to be involved in stress responses in plants that don't fit in any of the signalling groups.	Dehydrin, ERD3, SALI3, SLT1
Unknown	– Matched and hypothetical gene or domain – Expressed but of unknown function	

using DNAse I from the same manufacturer to remove genomic DNA traces. First strand cDNA was synthesized from 2 µg total purified RNA by reverse transcription. One µl of 10 mM dNTPs and 1 µl of oligo dT (25 mer) was added to each RNA sample. RNA was then denatured at 65°C for 5 minutes, chilled on ice for 10 minutes, and quickly centrifuged. Ten µl of a master mix was added to each reaction containing 4 µl 5X First Strand Buffer, 1 µl RNAse OUT, 2 µl 10mM MgCl2, 2 µl 0.1mM DTT, and 1 µl (200 units) of SuperScript III (all from Invitrogen, Carlsbad, CA) per sample. The reaction was incubated at 50°C for 1 hr followed by 15 min at 70°C to inactivate the reaction. RNA complementary to the cDNA was removed by adding 1 µl (2 units) of RNAse H (Invitrogen) and incubating at 37°C for 20 min. The final reaction mix was diluted with 180 µl of nuclease-free water. The cDNA concentration was measured using a NanoDrop ND-1000 spectrophotometer and adjusted to 30 ng/µl. The PCR was run with 2 µl of cDNA template, 7.5 µl water, 0.8 µl of each primer at 4 µM, and 8.9 µl of SYBR Green master mix (Invitrogen) with ROX as reference Dye. Primers were designed using Primer3 Software v. 0.3.0 (Whitehead Institute for Biomedical Research, Cambridge, MA). All primers used are displayed in Table 2. The reaction (20 µl) was used to quantify the cDNA by amplification using the MX3000P QPCR system (Stratagene, Kirkland, WA). A total of 13 genes were tested to confirm microarray results. Three constitutive controls were initially selected for normalization, a soybean β-actin and the soybean constitutive controls cons6 and cons15 (Libault et al., 2008). The constitutive control cons15 (homology to a CDPK) was found to be more consistent than β-actin for this study. Therefore, results presented for qRT-PCR are relative to cons15 only. PCR products were sequenced to verify that the correct amplicon was being produced.

Batch BLAST for Identification of Fungal Genes Cross-Hybridizing to Soybean cDNAs

BLASTn was run as batches using the "blastall" program from NCBI on a local server. The *S. sclerotiorum* genome sequence ("transcripts.fasta" data set) was downloaded from the *Sclerotinia sclerotiorum* Sequencing Project at the Broad Institute of Harvard and MIT (http://www.broad.mit.edu) as the reference database. EST sequences from both 5′ and 3′ ends of soybean cDNAs that comprise our list of significant genes in both time and genotype studies were BLAST against the *S. sclerotiorum* genome. Results were parsed for top hit information using PERL programs.

Batch BLAST of Soybean Markers for Resistance to *S. sclerotiorum* and Microarray cDNAs to the Soybean Genome for In-Silico Mapping

The soybean 7x genome (Soybean Genome Project, DOE Joint Genome Institute http://www.phytozome.net/soybean) was downloaded and formatted as a reference database. Seven SSR markers for QTLs for resistance to *S. sclerotiorum* that were previously identified in at least three populations from the study of Arahana et al. (Arahana et al., 2001) were selected and their sequences retrieved from NCBI. SSR marker sequences were BLAST against the soybean 7x genome using the "blastall" program from NCBI on a local server to obtain their position on the soybean genome scaffolds. Similarly, the available EST sequences from both 5′ and 3′ ends from cDNAs selected as highly significant in the present microarray study were BLAST against the soybean genome to identify locations within sequence scaffolds. Locations of markers were then compared to locations of cDNAs to identify those located to the same scaffold and at most 500 kb away of each other.

RESULTS AND DISCUSSION

Differences in the rate of lesion development were clearly visible between R and S genotypes over 5 days. Infection on stems of resistant PI 194639 seedlings progressed more slowly compared to that of the susceptible cultivar Williams 82. The infection front in the stems of PI 194639 accumulated red coloration, most likely resulting from an accumulation of phenolic compounds (Fig. 2). To capture differential gene expression of R and S, we monitored transcript levels early during the infection

Table 2. Primers used for qRT-PCR.

Microarray clone ID	Annotation	Study	Forward primer	Reverse primer
Gm-r1089-8081	chloroplast membrane	genot	gcatgtgctttcctgctcct	cagatcgacggcccagaat
Gm-r1021-3690	Csf-2	genot	ggtaggcaccaatttctttgctc	aggaaaagggaagcgttgtg
Gm-r1088-2632	CYP82A3	genot	tggagtagccttggtgttgg	ccctttggaagtgggagaag
Gm-r1083-1013	homolog to R gene	genot	tggaacaccaaaccaaacc	tggcattgtgagaggctagag
Gm-r1070-5331	SF21C2 protein	genot	acaagccaccgcttatcacg	gtggagcagcaatgaacagg
Gm-r1089-3849	transcription factor	genot	aaaggggtgttctcattgtcc	gagggaactatacccagaaggaag
Gm-r1083-517	unknown	genot	ccataacaccaacagcaccac	ccaaaagggataaggggtaggac
Gm-r1070-2321	β-1,3-glucanase	time	tgctgtgaaaagagcataggg	ttccttcaggggtgatgtgag
Gm-r1089-4085	class I chitinase	time	ggccaccgttgattatgttg	gacagccctatggttttggatg
Gm-r1088-8177	metalloproteinase	time	atgcgagtttggcagcagag	tcacagtcagagcaacgaaagg
Gm-r1088-8829	PR-1	time	tgacacagttgcggctttg	tccccgtattttccatcacc
Gm-r1089-8255	class I chitinase	time	ggccaccgttgattatgttg	gacagccctatggttttggatg
Gm-r1089-8595	unknown	time	gcacctttcgttgctctgac	gacttggagactgtggcgatg
Gm-c1026-2067†	CDPK-related kinase	cons15	taaagagcaccatgcctatcc	tggttatgtgagcagatgcaa

†Constitutive control used for data normalization (Libault et al., 2008).

process. The time points selected were based on preliminary pilot studies which showed that changes in expression levels were difficult to detect using earlier time points (Vuong and Calla, unpublished data, 2005). For simplicity in discussion, a spotted cDNA is assumed to represent a gene and the term "gene" is used interchangeably with spotted cDNA, and only genes determined to be statistically significant are discussed.

Samples from R and S, at 8 and 14 hpi, were used in pairs to hybridize cDNA microarray slides (Fig. 1, panel A). Statistical analysis identified 1270 genes (FDR corrected p-value <0.05) differentially expressed as disease progressed from 8 to 14 hpi. The direction of differential expression in this time-course study was the same for all selected genes across both genotypes (either up or down) (See Supplemental Table 1). For that reason all the data concerning time changes is presented together across both varieties. Direct comparison of R versus S interactions identified 105 differentially expressed genes using the same criterion (FDR p-value <0.05); similarly as in the time-course analysis, the direction of differential expression between the S and R genotypes was the same for all genes at both time points (See Supplemental Table 2). These results indicate that the changes in gene expression that occurred as the disease progressed during this early stage of pathogen infection were more robust and easier to assign significance than the differences observed between the R and S genotypes. Genes that were found to have different expression patterns across varieties are most likely the ones directly or indirectly related with specific susceptibility/resistance outcomes, while genes having differential expression across time points are most likely general responses of the plant to the infection that may or may not lead to enhanced resistance.

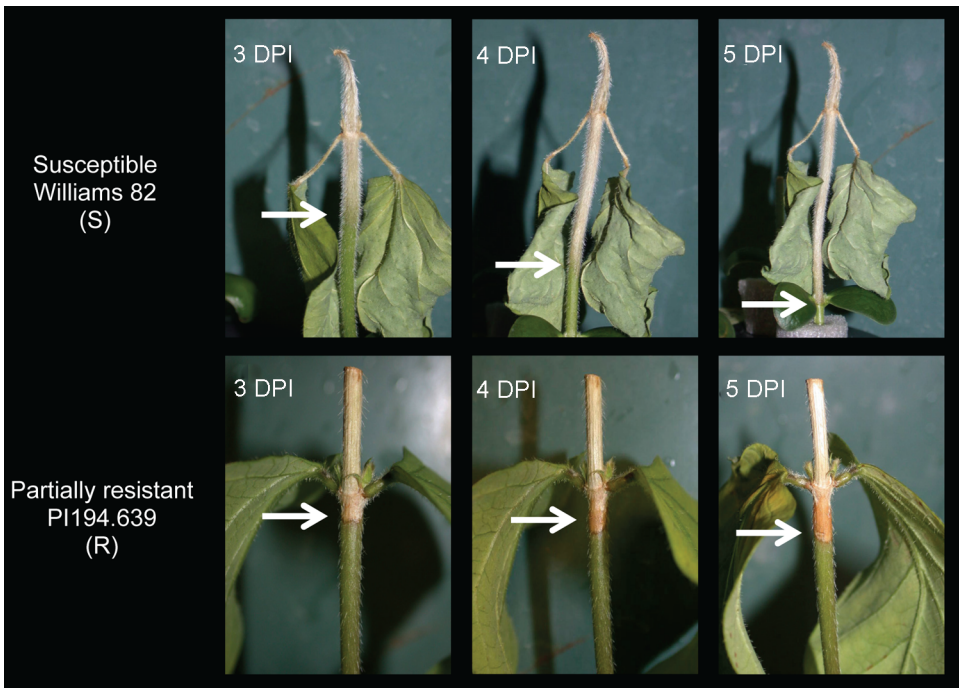

Figure 2. Differential response observed in soybean plants to *S. sclerotiorum* infection. Williams 82 (S) is a fully susceptible genotype and PI 194639 (R) shows partial resistance. Fifteen-day-old plantlets were inoculated with growing mycelia on cut stems. The infection progression was faster in S compared to R; arrows point to the infection front. Red coloration is observable at the infection front of R. DPI = days post inoculation.

Functional Classification of Differentially Regulated Soybean Transcripts upon Infection of Stems with *S. sclerotiorum*

The 1375 cDNA that showed differential expression during this study were classified into functional categories. EST sequences available for the spotted genes were BLAST against public databases and the annotations were used to assign functional categories (Table 1). The comparison between plant entries and the comparison between time points yielded fairly similar results in terms of percentage of genes classified into each of the categories (Fig. 3). In both analyses, the sum of genes in the categories "no hits," "miscellaneous," and "unknown" accounted for almost half the genes (46% of the genes in the time-course analysis and 49% in the comparison between varieties). Signaling genes and genes related to DNA/RNA processing had lower percentages in the comparison between varieties, but genes related to primary metabolism and protein synthesis and processing were similar. Genes related to oxidative processes had a much smaller percentage in the comparison between time points, accounting for only 3% (39/1270) in the time analysis and almost 6% (6/105) in comparison between R and S. There were no genes with significant differential abundance in the cell-wall category for the R vs. S comparison, whereas cell-wall related genes accounted for more than 2% (30/1270) of the differentially expressed genes in time-course analysis.

These results indicate that the microarray experiment successfully identified genes modulating during this very early stage in the plant response to inoculation that were related to signaling events, transcript level regulation/processing, protein trafficking and modification, and modulation of the oxidative state, in addition to the changes in transcript levels related to secondary metabolite production, all of which are events often observed in plant defense. Following is a description of gene expression patterns of interesting groups of genes and a discussion on their probable involvement in soybean response to the pathogen.

Soybean Genes Changing in Abundance over Time during Early Infection with *S. sclerotiorum*

Cell-Wall Genes

The time-course analysis identified 30 genes changing in expression from 8 to 14 hpi that were related to cell-wall modification. Among this group were enzymes involved in the formation of cellulose, hemicellullose, and pectin (Table 3). Although the majority of these gene transcripts were reduced in abundance, genes related to structural proteins such as expansins, increased at 14 hpi compared to 8 hpi. Expansins are a large group of non-enzymatic cell-wall proteins with several functions, the main one being cell expansion (Li et al. 2003). Expansins are also involved in cell-wall disruption and softening, events that can be observed during soybean infection with *S. sclerotiorum*. Although there is a recent report on expansins being up-regulated in transgenic *Arabidopsis thaliana* expressing a pathogenesis-related (PR) protein PR-10 from pea (*Pisum sativum* L.) (Krishnaswamy et al., 2008), we are not aware of any direct link between expansins and plant defense against fungal pathogens. Expansins were not among the 105 genes showing differential expression between R and S, suggesting that these proteins may be working in a general response to pathogen infection, perhaps enhancing wall softening to the advantage of the pathogen.

Defense-Related Genes

As a clear indication that the soybean tissue initiated a defense response against the invading pathogen in both R and S, 42 transcripts were identified with high homology to defense-related genes with differential abundance in time, accounting for about 5% of the known genes (disregarding the ones with no hits and unknown functions). Consistent with a more active defense response as infection progressed, 36 out of the 42 genes were highly increased at 14 hpi relative to 8 hpi (Supplemental Table 1).

Genes differentially regulated in the defense category included several apoptosis-related, putative R genes with LRR domains, and homologs to genes encoding PR proteins PR-1, PR-2, PR-5, and PR-10. Differential regulation of apoptosis-related genes in time may support the hypothesis of oxalic acid triggering programmed cell death (PCD) (Kim et al., 2008); however, our study did not directly test this association. R gene induction may give insight into new defense hypotheses as there are no known R genes working in the interaction with *S. sclerotiorum* or other necrotrophic fungal pathogens. In contrast, PR genes are very well known to be induced upon defense of a wide array of fungal pathogens. Nine PR transcripts were found to be highly increased in abundance at 14 hpi compared to 8 hpi in both R and S (Supplemental Table 1). Of these nine transcripts, five were homologs of PR-5 (proteins include thaumatins, thaumatin–like proteins and osmotin) and are known to have antifungal properties in plants (Abad et al., 1996; Hejgaard et al., 1991; Vigers et al., 1992). Several transgenic approaches have been conducted to confer resistance to fungal pathogens by expressing PR-5 transgenes (Liu et al., 1994; Velazhahan and Muthukrishnan, 2003). Beyond the potential role of thaumatin as an anti-fungal substance produced by soybean, another important significance of this result is that it serves as an indicator of the sensitivity of the microarray analysis to detect transcripts with differential abundance in response to the early infection process of *S. sclerotiorum*.

Signaling Genes

A major goal of this study was to identify genes related to early signal transduction in response to infection. We succeeded in this goal by identifying numerous genes across a variety of signaling pathways including those related to GTP-binding proteins, inositol, and ethylene.

G Proteins and GTP-binding Proteins

Six genes with homology to GTP signaling-related proteins were found differentially regulated at 14 vs. 8 hpi: two G-protein coupled receptors (GPCR) and four G-proteins (GTP binding proteins). Two genes corresponding to G-proteins increased and two decreased in transcript levels in the time-point comparison; the result was the same for both genotypes (Supplemental Table 1). Recent studies in plants have demonstrated direct roles for G-proteins in plant defense responses such as in the production of reactive oxygen species (ROS) and the activation of NADPH oxidases (Park et al., 2000), ion channels, and phospholipases (Meijer and Munnik, 2003). A heterotrimeric G-protein in *Arabidopsis thaliana* was demonstrated to play a role in the jasmonate-mediated signaling response to the necrotrophic pathogen *Alternaria brassicola* (Trusov et al., 2006). Additionally, the production of several phytoalexins and secondary metabolites were also associated to G-proteins in various plant species (Kurosaki et al., 2001; Roos et al., 1999; Zhao and Sakai, 2003). This study provides additional material in support of G-protein association with defense responses in soybean (Supplemental Table 1).

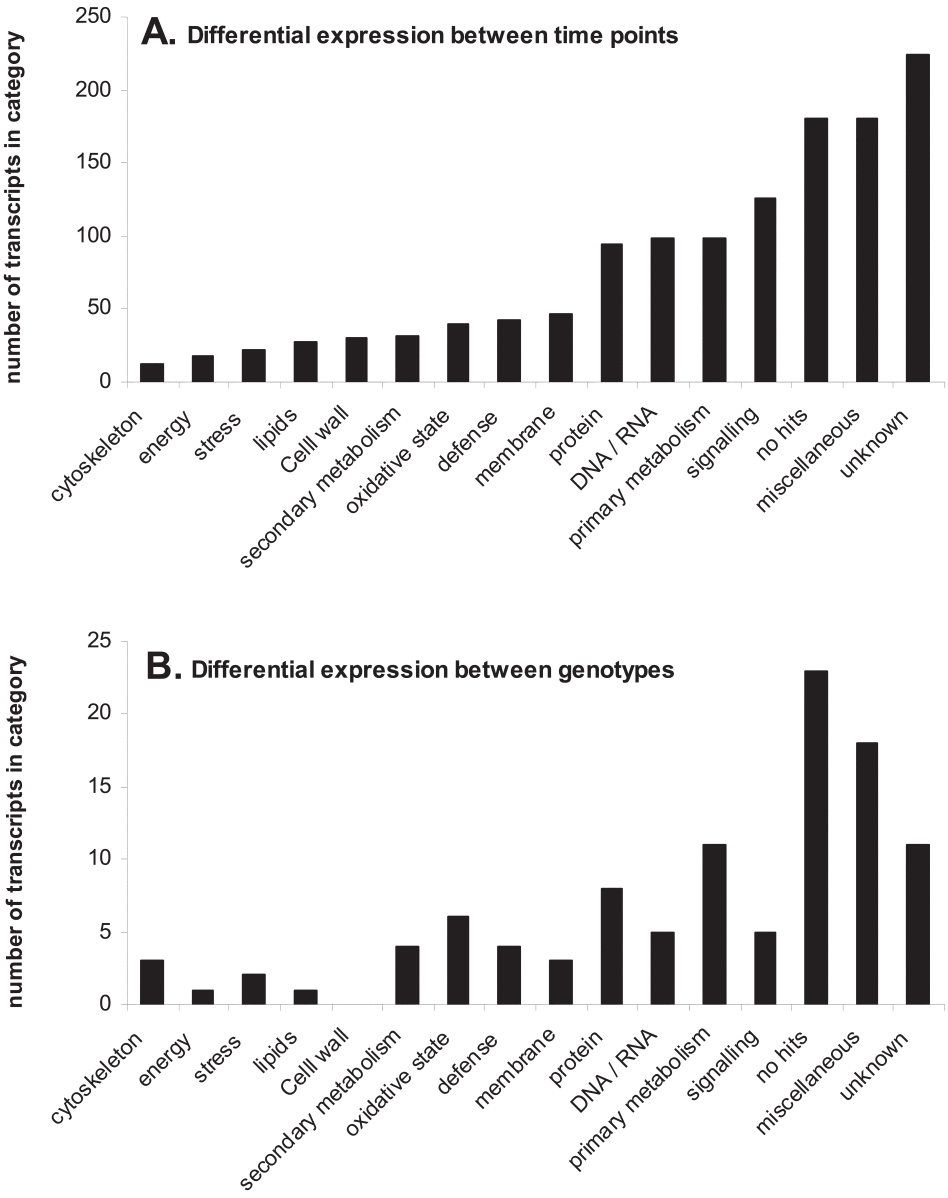

Figure 3. Functional classification of differentially expressed genes. Genes with FDR corrected *p*-value <0.05 were classified into functional categories using sequence homology to entries in public databases. Panel A. Genes with statistically significant change in abundance in the comparison between time points on soybean plants inoculated with *S. sclerotiorum*. Panel B. Genes with statistically significant difference in abundance between genotypes.

Inositol Signaling Pathway

Myo-inositol phosphate synthase is involved in the first committed step for de novo biosynthesis of inositol from glucose-6-phosphate. Inositol has many roles in plants including the phosphatidylinositol signaling pathway, auxin storage and movement in the plant, production of stress-related molecules, cell-wall biosynthesis, and phytic acid biosynthesis (Loewus and Loewus, 1983). The role of inositol in the biosynthesis of the plant cell wall is essential. Inositol can be oxidized to UDP-glucoronic acid, and this oxidized form of inositol could provide an alternative pathway for the synthesis of UDP-sugars (Loewus and Murthy, 2000) which are substrates for cell-wall polysaccharide synthesis. As mentioned above, our study identified several cell-wall synthesis and modification-related transcripts, including UDP-sugar associated enzymes that were differentially expressed with statistical significance upon infection with *S. sclerotiorum*, supporting the hypothesis of the central role of the cell wall in the interface between this pathogen and host. Legendre et al. (1993) reported that the phophatidylinositol lipid turnover pathway was linked with the stimulation of H_2O_2 production by elicitor molecules in soybean plants. Additional studies in pea, tobacco (*Nicotiana tabacum* L.), tomato (*Solanum lycopersicum* L.), soybean, and lucerne (*Medicago sativa* L.) showed increases of cellular inositol 1,4,5-trisphosphate (IP3) following treatment of cells

Table 3. Cell wall-related genes with differential regulation in time-course analysis (14 vs. 8 hpi).

Microarray clone ID	Fold change†	*p*-value‡	Fold change†	*p*-value‡	Sub category	Annotation
Gm-r1021-1490	−0.362	0.025	−0.228	0.130	Cell adhesion	beta-Ig-H3/fasciclin-Arabinogalactand protein
Gm-r1083-3766	−0.671	0.033	−0.436	0.140	Cell adhesion	fasciclin-like arabinogalactan-protein 2
Gm-r1021-2754	−0.694	0.027	−0.619	0.062	Cell adhesion	fasciclin-like arabinogalactan-protein 7
Gm-r1070-7235	0.830	0.027	0.548	0.123	Callose	callose synthase
Gm-r1089-4947	−1.144	0.028	−0.692	0.140	Cellulose	biosynthesis/ cellullose synthase
Gm-r1083-683	−0.826	0.023	−0.732	0.059	Cellulose	degradation/ Endo-1,4-beta-glucanase
Gm-r1070-2226	−0.552	0.037	−0.429	0.110	Cellulose	biosynthesis/ cellulose synthase catalytic chain
Gm-r1089-3965	−0.548	0.044	−0.690	0.031	Cellulose	degradation/ Endo-1,4-beta-glucanase
Gm-r1021-1934	1.493	0.020	1.175	0.069	Cellullose	biosynthesis/ UDP-glucose:protein transglucosylase
Gm-r1089-396	−1.162	0.005	−0.445	0.104	Hemicellullose	xyloglucan endotransglycosylase
Gm-r1088-8463	−0.591	0.034	−0.430	0.101	Hemicellullose	xyloglucan endotransglycosylase
Gm-r1088-4246	−1.515	0.012	−1.089	0.050	Hemicellullose	xyloglucan endotransglycosylase
Gm-r1070-6513	−0.894	0.023	−0.435	0.200	Pectin	pectin methylesterase
Gm-r1070-5102	−0.809	0.007	−0.424	0.077	Pectin	pectin methylesterase
Gm-r1089-298	−0.528	0.021	−0.415	0.059	Pectin	polygalacturonase
Gm-r1083-188	−0.491	0.015	−0.336	0.075	Pectin	nucleotide sugar epimerase-like protein
Gm-r1088-5667	0.333	0.061	0.431	0.039	Pectin	peptidoglycan biosynthesis
Gm-r1083-3987	1.109	0.046	1.000	0.093	Pectin	pectinesterase
Gm-r1021-3627	1.897	0.008	1.633	0.031	Pectin	polygalacturonase inhibiting protein
Gm-r1088-1103	−0.762	0.018	−0.401	0.145	Structural proteins	expansin
Gm-r1088-3031	1.089	0.027	0.692	0.121	Structural proteins	expansin
Gm-r1088-5190	1.201	0.006	0.575	0.067	Structural proteins	expansin
Gm-r1021-2732	1.293	0.038	1.326	0.060	Structural proteins	expansin
Gm-r1089-3338	1.500	0.038	0.920	0.167	Structural proteins	expansin
Gm-r1088-5055	1.572	0.014	0.447	0.366	Structural proteins	expansin
Gm-r1089-8201	1.797	0.043	1.479	0.089	Structural proteins	expansin
Gm-r1089-8696	2.431	0.021	2.634	0.028	Structural proteins	expansin
Gm-r1070-4170	−1.617	0.005	−1.395	0.022	Structural proteins	glycine-rich protein precursor (GRP)
Gm-r1070-571	−1.001	0.028	−0.822	0.077	Structural proteins	proline-rich protein
Gm-r1021-3212	−0.571	0.023	−0.536	0.053	Structural proteins	hydroxy-proline rich glycoprotein (HPRG)

†Fold change expressed as $\log_2$ of the 14:8 hpi intensity ratio.

‡FDR corrected p-value.

with elicitors (Kamada and Muto, 1994; Legendre et al., 1993; Walton et al., 1993).

Seven genes coding for myo-inositol phosphate synthases were observed to significantly decrease at 14 hpi compared to 8 hpi. Based on sequence matches to the TIGR TC contigs, six of the seven genes related to myo-inositol phosphate in the time-course analysis appear to code for the same protein, possibly the result of multiple, highly similar gene family members. The other five genes related to inositol signaling also decreased in abundance as time progressed, among them was one transcript annotated as a phosphatidylinositol transfer-like protein, two as phosphatidylinositol kinases, and two phospholipases. The significance of the reduction in abundance of inositol-related genes in time is unclear. Their gene expression is either higher at 8 hpi (relative to 14 hpi) to elicit rapid defense, or this is possibly a mechanism used by the pathogen to suppress defense for enhanced infection of the tissue.

Ethylene Signaling Pathway

Ethylene (ET) is a modulator of many mechanisms in plants, including growth, development, and response to biotic and abiotic stresses. Several genes involved in the ET signaling pathway have been cloned and characterized in *Arabidopsis thaliana* via mutants with defective ethylene responses. A putative signaling pathway has been proposed using the detected major components (Stepanova and Ecker, 2000). Additionally, the ET signaling pathway has been shown to interact either positively or negatively with the salicylic acid (SA) and jasmonate (JA) pathways in defense responses (Lorenzo et al., 2003; Wang et al., 2002).

Berrocal-Lobo et al. (2002) demonstrated that constitutive expression of ERF1 (ethylene responsive factor1) in *Arabidopsis thaliana* increased the resistance of the plant to *Botrytis cinerea* and *Plectosphaerella cucumerina*, both necrotrophic fungal pathogens. ERF1 was also implicated in the integration of the JA with ET pathways (Lorenzo et al., 2003). An ERF1 was also found

to be significantly up-regulated in *S. sclerotinia*-inoculated *B. napus* when compared to mock-inoculated plants (Zhao et al., 2007). However other ERFs were not confirmed to be directly regulated by ET and appeared to be responsive to other stimuli (Wang et al., 2002).

ET is synthesized from S-adenosyl-L-methionine (S-AdoMet) via 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. ACC is oxidized by ACC oxidase to yield ethylene, carbon dioxide, and cyanide. The cyanide produced is then detoxified by β-cyanoalanine synthase (Kende, 1993). The time-course analysis on our data identified four genes with increased transcript levels involved in ET biosynthesis: three ACC oxidases and one β-cyanoalanine synthase. Several other ERFs and one ERS (ethylene response sensor homologs) were identified, all showing increased expression at 14 hpi compared to 8 hpi (Table 4), indicating, consistent with the above mentioned studies, that ethylene is critical in the plant defense reaction against this necrotrophic fungus, and further supports the validity of the microarray data from the present study.

Table 4. Ethylene biosynthesis and ethylene-related factors changing in expression at 14 vs. 8 hpi.

Microarray clone ID	Williams 82 (S)		PI 194.639 (S)		Annotation
	Fold change†	*p*-value‡	Fold change†	*p*-value‡	
Gm-r1088-4339	1.089	0.033	0.908	0.068	EIN3-binding F-box protein 1
Gm-r1021-4003	0.754	0.016	0.690	0.046	ERF / APETALA related
Gm-r1089-8513	1.900	0.017	1.948	0.028	Ethylene response factor ERF1
Gm-r1083-4514	0.821	0.037	0.675	0.095	Ethylene response sensor
Gm-r1083-2406	0.165	0.064	0.252	0.042	Ethylene transcription factor
Gm-r1070-903	0.727	0.030	0.643	0.070	Ethylene-responsive element binding factor
Gm-r1088-5816	1.037	0.017	0.670	0.083	ACC-oxidase
Gm-r1021-612	1.755	0.015	1.038	0.103	ACC-oxidase
Gm-r1021-2425	0.266	0.191	0.763	0.031	ACC-oxidase
Gm-r1083-4527	2.122	0.017	1.714	0.058	Beta-cyanoalanine synthase

†Fold change expressed as $\log_2$ of the 14:8 hpi intensity ratio.

‡FDR corrected *p*-value.

Secondary Metabolism-Related Genes

A group of genes that is of special importance in defense are those involved in secondary metabolism. This study identified 32 genes belonging to the secondary metabolism category changing in expression across the 8 to 14 hpi time points. These genes were further separated between those of the phenylpropanoid pathway and those of other secondary metabolism pathways.

Phenylpropanoid Biosynthesis

The study comparing 14 to 8 hpi detected 23 genes coding for enzymes in the phenylpropanoid pathway being differentially expressed. Genes in this pathway were classified into three sub-pathways based on Zabala et al. (2006) (i) isoflavonoids, (ii) flavonoids and anthocyanins, and (iii) lignin biosynthesis (Fig. 4). Transcripts coding for enzymes in the isoflavonoids pathway seemed to be mainly increasing in abundance at 14 hpi compared to 8 hpi. Although no isoflavone synthase (IFS) genes were identified as differentially expressed, five isoflavone reductase homologs (IFRs) increased in abundance in both genotypes. The average fold change of IFR in S was 3.9 while R was 3.24. IFRs are the enzymes directing the synthesis of isoflavanones from isoflavones (Fig. 4). The results suggest that plants synthesize increased amounts of isoflavanones in dynamic response to *S. sclerotiorum* infection.

Transcripts of genes belonging to the flavonoid pathway were not detected as significantly changing in abundance in this study. A study on the pattern of expression of the phenylpropanoid pathway in soybean infected with *Pseudomonas syringae* pv. glycinea, suggested that induction of the flavones pathway was characteristic of R-gene mediated defense responses (Zabala et al., 2006; Zou et al., 2005). Consistent with this idea, the response of soybean *to S. sclerotiorum* apparently does not affect the flavonoid pathway and is a non-R-gene dependent response.

Leucoanthocyanidin dioxygenase (LDOX) is the key enzyme leading to the synthesis of anthocyanins (Fig. 4). Two transcripts annotated as LDOX and LDOX-like protein were found to be decreasing in abundance at 14 hpi compared to 8 hpi in both R and S. However, two homologs involved in the glucosylation of flavonol precursors to produce the first anthocyanin derivatives were up-regulated in both varieties. A study performed by Coley and Aide (1989) suggested that very young leaves in tropical forests that often present red and purple coloration have increased levels of anthocyanins that, hypothetically, provide protection to leaves from pathogens until they have fully developed cuticles and lignin. Lorenc-Kukula et al. (2005) studied the effect of ectopic expression of one of the glucosyl-transferases in the anthocyanin production pathway in potato that leads to increased resistance against the bacterial pathogen *Erwinia carotovora*. Anthocyanins are known to be antioxidant molecules due to their strong redox capacities related to the ability of polyphenol-derived radicals to stabilize and delocalize unpaired electrons, and from their ability to chelate transition metal ions (Rice-Evans et al., 1997).

Our results indicate that there is modulation in levels of transcripts coding for enzymes of the anthocyanin pathway, however this modulation varied. It is possible that at early time-points LDOX expression increased and later decreased (explaining the negative fold change in LDOX) to divert resources to the production of pathway end-products. The exact role of anthocyanins in defense is not clear but it could be that these compounds neutralize damaging ROS induced by oxalic acid.

The time-course analysis revealed 11 differentially expressed genes related to lignin, five of them being

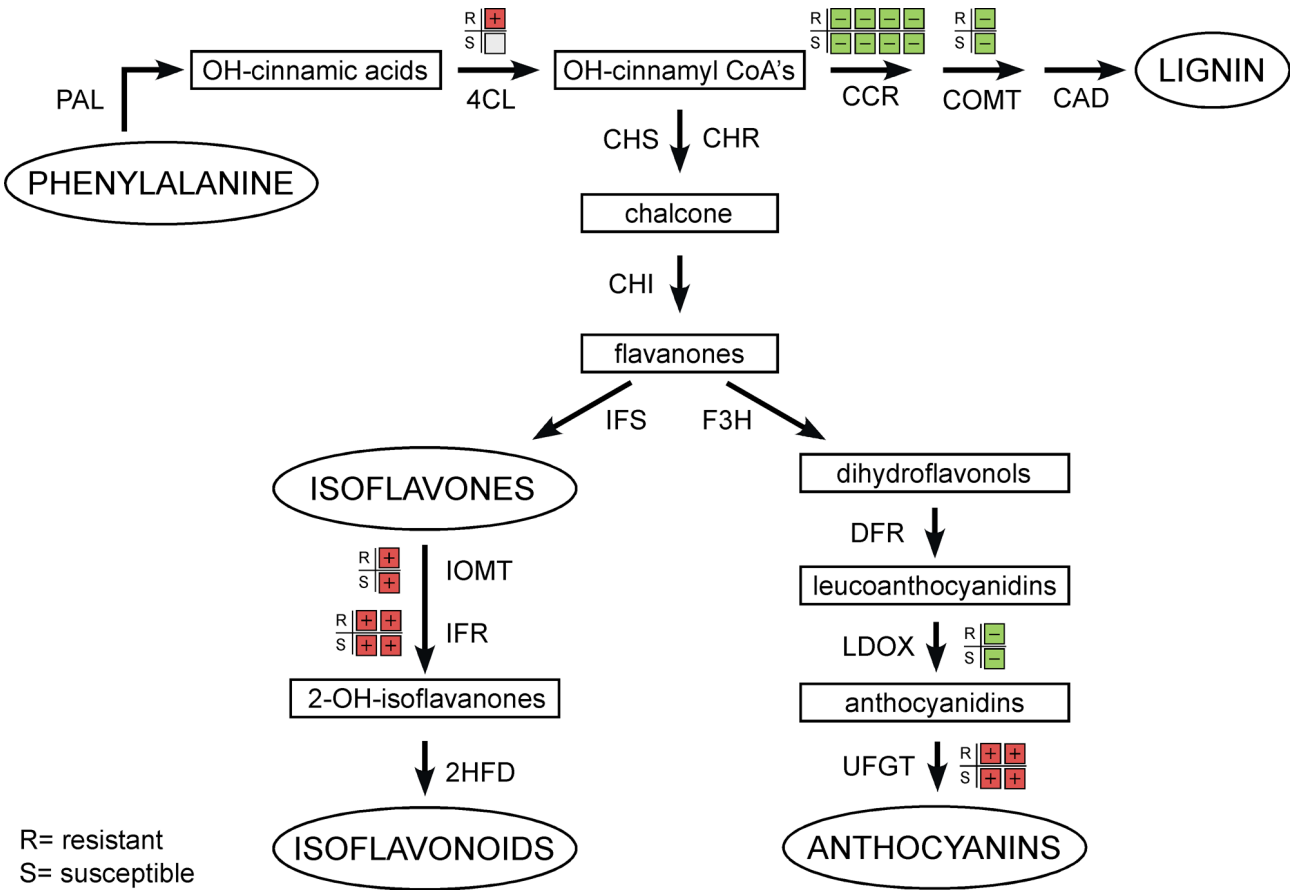

Figure 4. Phenylpropanoid pathway. Changes in abundance at 14 hpi as compared to 8 hpi of genes coding for enzymes in the phenylpropanoid pathway in soybean challenged with *S. sclerotiorum*. Squares represent individual cDNAs. Red color represents increased expression levels in 14 vs. 8 hpi, green decreased, and grey no change.

homologs to cynnamoyl CoA reductase (CCR), the enzyme responsible for diverting the phenylpropanoid pathway into the branch of lignin synthesis. The CCR genes all decreased over time. Additionally, a caffeic acid O-methyltransferase homolog (COMT), another key enzyme in the lignin pathway, also decreased over time. Three of the increased genes in lignin biosynthesis were homologs to laccase-like proteins, enzymes widely found in fungal pathogens to aid the de-lignification of plants through oxidation. Plants also have laccases but their exact role is unclear (Mayer and Staples, 2002). The present study shows strong evidence that the plant might be diverting substrates from the lignin pathway to increase production of isoflavonoids and anthocyanins (Fig. 4). The present study also supports the findings on lignin studies by Peltier et al. (2009).

Soybean Genes with Differential Expression between R and S during Infection with *S. sclerotiorum*

One of the important goals of this microarray study was to identify gene modulations reflective of putative plant defense processes in soybean leading to partial resistance to *S. sclerotiorum*. As mentioned previously, a set of 105 genes were detected as having significant differential expression between R and S genotypes with a strict cut-off p-value (FDR corrected p-value <0.05) (Supplemental Table 2). Of those genes, 53 had more abundance in the R genotype and 52 had more abundance in the S genotype. Both groups are considered of importance and to be the best candidates for explaining the partially resistant genotype. Using a stringent p-value may result in many important genes being missed (false negative) but the benefit was that the selected genes have a higher degree of confidence in being truly different in abundance.

As mentioned, half (53) of the genes that were differentially expressed between R and S increased in R compared to S. Three genes in this group belong to the cytoskeleton category: an actin, a kinesin, and a dynamin. Plant cells are known to relocate intracellular constituents upon infection of pathogens and this relocalization is guided by cytoskeleton components (Takemoto and Hardham, 2004). Actin filaments play a key role in the formation of cytoplasmatic aggregation preceding papillae formation, and papillae formation is one of the first responses to pathogen attack as a basal defense mechanism (Aist, 1976). This result suggests that R plants might be able to react faster to pathogen ingression possibly by conducting cytoplasmatic aggregation in a more rapid or efficient manner as compared to the

S plants. Additional gene transcripts that were found to be significantly higher in abundance in R compared to S included two transcription factors, one a homolog to a MYB-related transcription factor (VlMYBB1-1) and one homologous to a mini zinc finger-like protein. Two peroxidases were also significantly higher in abundance in the resistant tissue at both time points studied. Peroxidases not only play an important role as antioxidants but are also used by the cell as lignin-modifying agents through cross-linking of cell-wall components. Peroxidases were found to increase in levels in a proteomic study in *B. napus* infected with *S. sclerotiorum* (Liang et al., 2008) Among the secondary metabolism genes with significantly higher abundance in R was a transcript annotated as soybean anthocyanidin synthase, supporting the idea that anthocyanin and anthocyanidins may play an important role in defense of soybean against *S. sclerotiorum* in the R genotype.

Constitutively Differentially Expressed Transcripts between Genotypes

Differential expression between R and S in our experiment may be due to inherent constitutive gene expression differences and not due to pathogen induction; therefore, a separate microarray study was performed on untreated control samples from both varieties (Fig. 1, panel B). The comparison between untreated R and S samples identified 19 genes with significant differential expression at our default FDR corrected cut-off *p*-value <0.05. Annotation using public databases and functional classification identified genes in several categories, including energy utilization, DNA/RNA processes, protein processing, and miscellaneous (Supplemental Table 3).

Three of the genes identified in the control study were also found in the genotype analysis (Supplemental Table 3), and thus might be of special importance in the inherent resistance of the plant to the pathogen. BLAST results against public databases for the three sequences overlapping the genotype study resulted only in one of them matching a known gene (Gm-r1088-1636, a calcium signaling protein). Given the phenotypic differences between both genotypes, it was expected that more genes would have been constitutively differentially expressed. It is possible that the stringent FDR correction of the *p*-values masked identification of some weakly differentially expressed genes. Additionally, the microarray may lack some genes specific to the R genotype.

QRT-PCR Confirms Microarray Results

Quantitative real time RT-PCR (qRT-PCR) was conducted on 13 of the genes identified by the microarray analysis as being significantly differentially expressed. All six genes selected from the comparison between time points showed high consistency with the differential expression patterns of the microarray results (Fig. 5, panel A). Seven genes that came from the comparison between R and S were used (Fig. 5, panel B). Although the results for these seven genes were generally consistent with the microarray results, we were unable to obtain results reflective of differential expression of Csf-2 coding transcript at 8 hpi and for a transcription factor gene at 14 hpi. The lack of consistent differential expression of Csf-2 and the transcription factor at all time points in qRT-PCR probably reflects our inability to design ideal primers or the natural biological variation that occurs during these very early points, as new biological replicates were included in the qRT-PCR analysis.

Identification of Fungal Genes Cross-Hybridizing to Soybean cDNA

As the microarray analysis involved the isolation of total RNA from infected tissue, followed by the florescent labeling of all polyadenylated RNA, it is possible that some cDNA spots fluoresced due to the hybridization of labeled fungal RNA. Genes coding differentially expressed transcripts might have originated from the pathogen and were present in the infected tissue used for RNA extraction. To rule out the possibility that polyadenylated fungal transcripts were labeled and hybridized to the soybean cDNAs printed in the slides to give false positives, a BLASTn was performed between all the genes from the *S. sclerotiorum* genome sequence project and all the soybean cDNA sequences present on the tables of significant genes in this study. There were 17 cDNA spots with *S. sclerotiorum* sequence homology (e-value <10-7) to EST sequences with a possibility of cross hybridization (Table 5). One transcript (Gm-r1083-1968) had high enough sequence identity [96.5% over 117 nucleotides (nt), with e-value 6e –51] to make us suspect that a fungal transcript was very likely to cross-hybridize and was therefore not considered when analyzing the data from our results. The other 16 cDNAs with some similarity to *S. sclerotiorum* sequence had marginal sequence similarity or fairly short lengths of sequence identity, and therefore, they were still included in the final gene lists but their microarray results should be interpreted with caution.

In Silico Mapping of Differentially Expressed Genes in R vs. S Lines and Comparison with Markers Associated with Resistance

Sequences of all the differentially expressed cDNAs found in this study were compared against the public soybean 7x genome sequence (Soybean Genome Project, DOE Joint Genome Institute http://www.phytozome.net/soybean) with the aim of finding exact positions of each of the microarray cDNAs on the specific soybean sequence scaffolds. Likewise, the positions on the soybean 7x genome sequence of seven SSR markers linked to *S. sclerotiorum*-resistance that were previously identified in at least three populations (Arahana et al., 2001) were obtained [Satt_109 (LG-O), Satt243 (LG-O), Satt_134 (LG-L), Satt143 (LG-L), Satt129 (LG-D1a), Satt147 (LG-D1a), and Satt424 (LG-A2)]. The positions of the cDNAs were then compared to those of the SSR markers.

The resulting comparisons showed that five of the seven markers have at least three microarray cDNAs at a

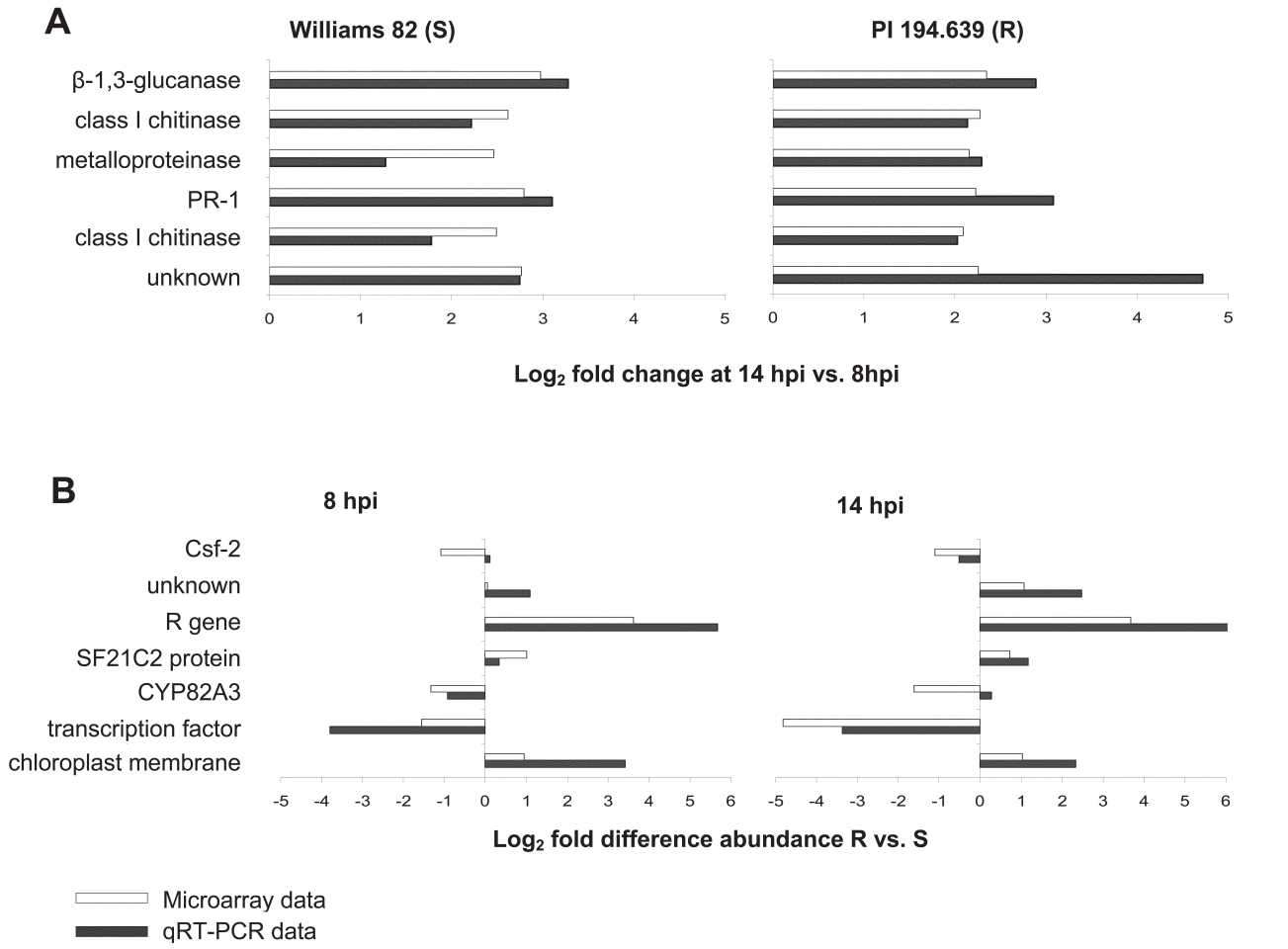

Figure 5. Quantitative real-time RT-PCR confirmation. QRT-PCR was used to confirm the accuracy of the microarray analysis. Panel A. Six genes showing increased abundance at 14 vs. 8 hour after inoculation with *S. sclerotiorum* in both genotypes: Williams 82 (S) and PI 194639 (R). Panel B. Seven genes with increased or reduced abundance in R vs. S at 8 and 14 hpi.

distance <500 kb. One marker (Satt134) did not have any cDNA mapping close to it, and another marker (Satt143) had only one cDNA within 500 kb. Interestingly, the two markers with no, or only one, closely located cDNA among the genes significantly differentially expressed in the microarray study were the only ones in the list of seven markers for which resistance was associated with escape mechanisms and not physiological resistance. Twenty-four genes selected as differentially expressed in the time-course analysis were located close to one of the other five markers (Table 6). Two markers (Satt129 and Satt147) were only 400 kb away from each other and thus, they have the same cDNAs listed as close hits. It is very possible that those two SSR markers map the same defense-related locus. There were six cDNAs that were found located close to both markers: three PR-5s (thaumatin), a phospholipase-D gamma subunit, a calcium-binding EF-hand subunit, and a transcription factor. As mentioned previously, thaumatins are known antifungal compounds and phospholipase-D and EF-hands are well-known signaling proteins.

Of the 105 significantly differentially expressed genes in the direct comparison between R and S genotypes, only two were located close to a marker. Remarkably, one of them, annotated as anthocyanidin synthase, mapped <300 kb away from an *S. sclerotiorum* resistance marker (Satt147), supporting the hypothesis that one factor that may affect the differential response between S and R varieties is the activation of the synthesis of anthocyanin and anthocyanidin-related compounds. The other cDNA from the genotype analysis mapped <38 kb away to Satt424. This cDNA was also selected in the time-course analysis as being significantly changing in abundance. The positions of the nineteen genes identified as constitutively differentially expressed in the control samples were also obtained but none of them had a location close to a marker in less than 500 kb. Although promising, results on the in silico mapping should be interpreted with caution, as the markers most tightly linked to *S. sclerotiorum*-resistance QTLs are not mapped precisely, and this resistance mapping has been inconsistent across genotypes and environments (Brian Diers, personal communication, 2008).

CONCLUSIONS

Soybean cDNA microarrays were used to identify genes that were differentially regulated during a defensive response against the necrotrophic fungal pathogen *S. sclerotiorum*. Despite some drawbacks in the use of

cDNA microarrays, for example the inability to differentiate between highly homologous gene families and to account for post-transcriptional modifications such as alternative splicing and mRNA editing, this experiment confirms the relatively high accuracy with which this tool is capable of identifying differential abundance in mRNA.

The study effectively provided two lists of genes with the highest probabilities of playing a part in the soybean–*S. sclerotiorum* interaction. The first list consists of genes that changed in abundance between 8 and 14 hpi, a time period in which pilot experiments determined to be the earliest that could be reliably performed with the cDNA microarray platform used to detect differential expression. The second list consists of genes with different abundance in the partially resistant genotype compared to the susceptible at both 8 and 14 hpi. Further understanding and clarification of the significance of the data was obtained by analyzing untreated samples.

Table 5. *S. sclerotiorum* transcripts with high probability of cross-hybridization with soybean cDNAs present in the microarrays.

Microarray clone ID	Study	Soybean annotation	*S. sclerotiorum* annotation	e-value	Length of hit	Match
						%
Gm-r1083-1968	time	stress-induced gene	conserved hypothetical protein	6.00E-51	117	96.50
Gm-r1070-4163	time	actin	actin	2.00E-36	281	81.80
Gm-r1088-3643	time	beta tubulin	betatubulin	2.00E-30	139	87.00
Gm-r1070-7495	time	beta tubulin	beta tubulin	6.00E-29	140	86.40
Gm-r1070-894	time	actin-like	actin	7.00E-24	152	84.20
Gm-r1089-3867	time	40S ribosomal protein S19	40S ribosomal protein S20	2.00E-22	380	79.00
Gm-r1070-7963	time	beta tubulin	beta tubulin	5.00E-19	131	83.90
Gm-r1088-4542	time	beta tubulin	beta tubulin	7.00E-19	95	87.30
Gm-r1070-4376	time	calmodulin	calmodulin	5.00E-14	227	79.20
Gm-r1089-3621	time	DNA polymerase delta subunit	hypothetical protein	2.00E-13	119	83.10
Gm-r1070-4556	time	histoneH2	histoneH3	6.00E-13	113	83.10
Gm-r1070-7963	time	beta tubulin	beta tubulin	7.00E-12	83	85.50
Gm-r1083-2517	time	unknown protein	glyceraldehyde-3-phosphatedehydrogenase	2.00E-11	35	100.00
Gm-r1021-3330	time	40S ribosomal protein S11	conserved hypothetical protein	7.00E-10	72	86.10
Gm-r1070-7630	time	heat shock 70kDa protein	heat shock 70kDa protein	2.00E-09	140	80.70
Gm-r1083-4132	genot	ADP-ribosylation factor 1	ADP-ribosylation factor 1	2.00E-09	87	83.90
Gm-r1021-1771	time	heat shock 70kDa protein	heat shock 70kDa protein	6.00E-09	123	81.30

All the data was annotated using public databases. The annotation allowed for functional classification of the genes and ultimately the classification led to a deeper understanding of the possible events occurring during the disease, on the basis of literature available from previous studies involving single genes, gene networks, protein expression, biochemical pathways, and physiological changes that plants experience during disease and stress.

The time-course analysis detected three important events during pathogen ingression in both R and S: modification of the cell wall, activation of signaling pathways, and synthesis of secondary metabolites. Modification of the cell wall seems to be characterized by an interesting increment in abundance of expansins for which a role in pathology is largely unknown. Detected signaling events comprise the widely reported increase in synthesis of ethylene and a less reported reduction in abundance of inositol signaling pathways. Secondary metabolite production concentrated on enhancement of isoflavonoid (isoflavones and isoflavanones) synthesis and also suggested an important role for the synthesis of anthocyanins. Although the main enzyme leading the synthesis of anthocyanins, LDOX, appeared to be reduced in abundance, enzymes acting in the later steps of the anthocynanin pathways were highly and consistently increased. This finding suggest that LDOX transcription might be induced at an earlier time point and that by 14 hpi transcription was focused on enzymes that catalyze the last glycosylation steps for the production of anthocyanins. Further data supporting the idea of increased anthocyanin and anthocyanidins synthesis is found in the comparison between genotypes where an anthocyanidin synthase had a twofold difference in R vs. S. It is noteworthy that, by reducing the strictness of the *p*-value used, to an uncorrected *p*-value <0.005, nine genes in the anthocyanin pathway (of 20 in the phenylpropanoid pathway) were modulating, and of those nine genes, seven were greater in abundance in R. The usefulness of the microarray study in this case is not limited to the identification of the synthesis of a specific compound, which can be done by other methods, but to the identification of the genes responsible for the synthesis of those anthocyanins and/or anthocyanidins in soybean including possible regulators, transcription factors and enzymes.

The comparison between R and S yielded a shorter list of 105 genes for which the differential expression was deemed significant. With so few genes available to analyze it is difficult to draw possible conclusions about the mechanisms used by the plants, but the genes selected are the primary candidates responsible for increased resistance of soybean to *S. sclerotiorum* in the PI 194639 (R) plants. QRT-PCR confirmed the result for 11 of these genes with high accuracy.

Results of this study extensively overlap with those from studies in *B. napus* infected with *S. sclerotiorum*. Peroxidases, for example, were found with increased abundance in the study comparing inoculated vs. noninoculated *B. napus* leaves (Yang et al., 2007). Evidence of peroxidase induction during *S. Sclerotinia* infection was recently confirmed in a proteomic study by the same group (Liang et al., 2008). This proteomic study not only

Table 6. Positions of SSR markers for resistance to *S. sclerotiorum* in soybean compared to positions of cDNAs with differential abundance (FDR $p<0.05$) in the microarray study. cDNAs mapping <500 kbp away from markers are shown.

	Marker: Satt_109; LG: 0; scaffold_52; position 3367602–3367834				
	Clone ID	cDNA position	Distance to marker†	Study‡	Annotation
cDNAs <500kbp away:	Gm-r1083-4362	3152671–3152887	215	time	endopeptidase
	Gm-r1083-2561	3817705–3818094	450	time	nitrate transporter
	Gm-r1021-683	3849345–3849897	482	time	putative hydrolase
	Gm-r1021-3489	3850178–3850448	482	time	putative hydrolase
	Marker: Satt243; LG: 0; scaffold_52; position 4921005–4921142				
	Clone ID	cDNA position	Distance to marker†	Study	Annotation
cDNAs <500kbp away:	Gm-r1070-5470	4473957–4474424	446	time	RNA polymerase II
	Gm-r1088-3248	4634104–4634386	286	time	phosphoglucomutase
	Gm-r1070-4196	4910296–4911097	10	time	homeodomain-related
	Gm-r1070-6363	5042383–5042773	121	time	elicitor receptor
	Gm-r1088-3643	5126743–5127893	205	time	beta tubulin
	Marker: Satt129; LG: D1a; scaffold_13; position 9484939–9485271				
	Clone ID	cDNA position	Distance to marker†	Study	Annotation
cDNAs <500kbp away:	Gm-r1088-8424	8905396–8905547	580	time	phospholipase D-γ
	Gm-r1083-4184	9104547–9105266	380	time	PR-5
	Gm-r1021-3935	9108461–9108843	376	time	PR-5
	Gm-r1083-1040	9108592–9108964	375	time	PR-5
	Gm-r1083-4829	9265037–9265394	219	time	calcium-binding EF-hand
	Gm-r1088-2660	9276566–9277069	207	time	transcription factor
	Gm-r1089-3625	9783919–9784565	298	time	phosphatidylinositol kinase
	Marker: Satt147; LG: D1a; scaffold_13; position 9150940–9151134				
	Clone ID	cDNA position	Distance to marker†	Study	Annotation
cDNAs <500kbp away:	Gm-r1088-820	8852510–8853241	298	genot	anthocyanidin synthase
	Gm-r1088-8424	8905396–8905547	245	time	phospholipase D-γ
	Gm-r1083-4184	9105294–9105550	45	time	PR-5
	Gm-r1021-3935	9108461–9108843	42	time	PR-5
	Gm-r1083-1040	9108592–9108964	42	time	PR-5
	Gm-r1083-4829	9265037–9265394	113	time	calcium-binding EF-hand
	Gm-r1088-2660	9276566–9277069	125	time	transcription factor
	Marker: Satt424; LG: A2; scaffold_22; position 2084752–2085179				
	Clone ID	cDNA position	Distance to marker†	Study	Annotation
cDNAs <500kbp away:	Gm-r1070-470	2122392–2122734	37	time	myo-Inositol-1-P-synthase
	Gm-r1070-470	2122392–2122734	37	genot	myo-Inositol-1-P-synthase
	Gm-r1089-7372	2504130–2504705	418	time	no hits

†Distance kilo base pairs.

‡Studies: conducted between R vs. S (genot) or 14 vs. 8 hpi (time).

identified the presence of the protein in infected tissue but also measured peroxidase activity during infection, demonstrating that it increased sharply after 24 hours of inoculation. The current study showed that peroxidase mRNA levels can be significantly increased well before 24 hpi. Genes of the ethylene pathway showed induction in all the studies in *B. napus* as well as in the present study on soybean. Several ERFs were found up-regulated in the Yang et al., 2007 study at the three time points. Zhao (Zhao et al, 2007) also found an ERF as well as ACC oxidase increasing in abundance between inoculated and control plants. A number of cell-wall structural and functional protein-encoding transcripts were also found significantly modulating in the *B. napus* study. Among those, the reduction on expression of a transcript annotated as xyloglucan endotransglycolase transcript is

consistent in the three studies when comparing susceptible genotypes. Similarly, in our soybean study, xyloglucan endotransglycolase was reduced in abundance in both time and genotype comparisons.

The present study tackles a difficult disease system to analyze. The in-silico mapping of the candidate genes in the soybean 7x genome provided an accurate comparison to serve as a starting point to identify key genes for future studies. The mapping provided precise data on the position of differentially expressed genes and relative positions to known markers for resistance of soybean to *S. sclerotiorum*. The results of the mapping provided additional support of the microarray analysis for several cDNAs mapping close to known markers, their putative role in defense is supported by the literature (i.e.: PR-5 and anthocyanidin synthase). The results suggest that two of the seven markers analyzed are markers for the same locus. The cDNA sequences located close to markers can also be used as new candidate genes for resistance and for marker development.

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